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Löfström, Charlotta; Hintzmann, Ann-Sofie; Sørensen, Gitte; Baggesen, Dorte Lau

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Highlights

- Outbreak of Salmonella ser. Typhimurium phage type DT41 in Danish broiler production
- 47 DT41 and RDNC isolates were analyzed with MLVA and PFGE
- 4 PFGE and 9 MLVA types were found; most common MLVA type 2-13-12-8-0212
- A spread from broiler breeders to broilers and slaughterhouse was documented

Outbreak of *Salmonella enterica* serovar Typhimurium phage type DT41 in Danish poultry production

Charlotta Löfström*, Ann-Sofie Hintzmann, Gitte Sørensen, Dorte Lau Baggesen

Division of Food Microbiology, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark

Running head: *Salmonella* Typhimurium DT41 in Danish poultry

* Corresponding author:
Dr. Charlotta Löfström, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark Tel.: +45 35 88 73 50, Fax: +45 35 88 70 01, E-mail: chalo@food.dtu.dk

Keywords: *Salmonella*, epidemiology, poultry, genotyping, MLVA, PFGE, DT41, outbreak

Abstract

Salmonella enterica subspecies *enterica* serovar Typhimurium (S. Typhimurium) is one of the most prevalent serovars in Europe - where both poultry and poultry related products are common sources of human salmonellosis. Due to efficient control programs, the prevalence of S. Typhimurium in Danish poultry production is very low. Despite this, during the past decades there has been a reoccurring problem with infections with S. Typhimurium phage type DT41 in the Danish poultry production without identifying a clear source. In the end of 2013 and beginning of 2014 an increased isolation of S. Typhimurium DT41 was noted mainly in this production, but also in other samples. To investigate this is in more detail, 47 isolates from egg layers (n = 5, 1 flock), broilers (n = 33, 13 flocks), broiler breeding flocks and hatches (n = 5; 2 flocks and 1 environmental hatchery sample), feed (n = 1), poultry slaughter house (n = 3, environmental sample and meat) were typed with multi locus variable number of tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE) to investigate the epidemiology of the outbreak. Based on PFGE results isolates were divided into four groups (Simpson´s index of diversity (DI) = 0.24 ± 0.15). Due to the low DI, PFGE was not sufficient to provide information to unravel the outbreak. Based on MLVA typing the DT41 - (42/47 isolates) and the RDNC isolates (5/47) were split into nine groups (DI = 0.65 ± 0.14). When a maximum divergence at one locus was permitted these could be gathered into four groups. Using this criterion, combined with epidemiological information, a spread of one type from broiler breeders to broilers and further to the poultry slaughter house was plausible. In conclusion, although it could be concluded that a spread within the broiler production pyramid had taken place the source of the sudden increase of *S.* Typhimurium DT41 remains unclear. To investigate this in more detail, further studies using whole genome sequencing to obtain a higher discriminatory strength and including isolates from a longer period of time and from various sources are in progress.

Introduction

Salmonella enterica subspecies *enterica* serovar Typhimurium (S. Typhimurium) is one of the most frequent causes of human salmonellosis in Europe (EFSA and ECDC, 2013), with poultry as an important reservoir (Mughini-Gras et al., 2014). The prevalence of *Salmonella* in poultry in Denmark is very low (Anonymous, 2014), but despite this, reoccurring isolations of particularly S. Typhimurium phage type DT41 (hereafter DT41), has been observed in broiler breeder flocks over the past decades (Litrup et al., 2010). DT41 has been isolated from e.g. poultry in different countries (EFSA and ECDC, 2013), wild birds (Pennycott et al., 2006), other animals (Davies et al., 2004; EFSA and ECDC, 2013) and poultry feed (Davies and Wales, 2010). These findings suggest a possibility of transmission between and within poultry flocks, as well as from the environment or via poultry feed (Horton et al., 2013).

Epidemiological characterization of isolates has the key aim to separate related and unrelated isolates, and different typing methods, e.g. phage typing, pulsed-field gel electrophoreses (PFGE) and multiple-locus variable number of tandem repeat analysis (MLVA) have been applied (reviewed by (Wattiau et al., 2011). No general rules for the determination of the optimal resolution and similarity threshold has been established as will depend on the actual bacterium of interest and its genetic nature (EFSA, 2013) For the analysis of typing data it is essential to include epidemiological data, and to balance the discriminatory power and threshold for separation in a way which gives the most meaningful grouping of isolates to obtain the highest level of epidemiological concordance (Struelens, 1996).

Previous studies using MLVA concluded that DT41 did not persist in Danish poultry production, but had an outside source (Litrup et al., 2010). It was speculated that a persisting clone could be genetically unstable, but this hypothesis could not be verified by *in-vivo* and *in-vitro* studies (Barua et al., 2013). During the end of 2013 and the beginning of 2014 an increase in the Danish poultry production was again noted for DT41. The aim of this study was to investigate the relation between isolates obtained during this time period

trying to establish a possible common source of the outbreak, using MLVA, PFGE and phage typing together with epidemiological information.

Materials and Methods

Salmonella strains and epidemiological information

S. Typhimurium strains were obtained from the strain collection at the Division of Food Microbiology, National Food Institute, Technical University of Denmark (DTU Food) and were collected through the Danish surveillance programs (Anonymous, 2014) during November 2013 - March 2014 (Table 1). Epidemiological information about the samples and links between production units were kindly provided by poultry industry partners, and supplemented with data from the Danish Herd Register (https://chr.fvst.dk). Isolates from broiler breeder flocks were obtained from flocks in production, and the age of the flocks was 50-56 weeks.

Serotyping and phage typing

Serotyping of *Salmonella* isolates was performed by molecular serotyping employing Luminex technology, as previously described (Fitzgerald et al., 2007; McQuiston et al., 2011), or by slide agglutination with polyclonal antisera (Statens Serum Institut, Copenhagen, Denmark), in accordance with the White – Kauffmann – Le Minor scheme (Grimont and Weill, 2007). Phage typing was performed in accordance with international standards (Callow, 1959; Anderson et al., 1977), as described by Public Health England (PHE), Colindale, London, UK. Isolates with reactions that do not confirm with the phage typing scheme were abbreviated RDNC.

MLVA and PFGE

The MLVA method developed by (Lindstedt et al., 2004) was performed as previously described (Torpdahl et al., 2007). PFGE was carried out according to the PulseNet protocol as previously described (Ribot et al., 2006) using *Xba*I (Fermentas, Lifesciences) as restriction enzyme.

Data analysis

Typing and strain metadata were entered into a Bionumerics v. 7.1 database (Applied Maths, Sint-Martens-Latem, Belgium) for further analysis. Cluster analysis was made for PFGE band patterns using a position tolerance of 1.5% and optimization of 1.5% and results were compared using the Dice coefficient for similarity and unweighted pair group method with arithmetic averages (UPMGA) for clustering.

MLVA allele numbers were analyzed in Bionumerics as character values, and minimum spanning trees (MST) were constructed using categorical coefficients and the Ward algorithm (Ward et al., 2009). The following priority roles were used to create networks: 1) Maximum number of N-locus variants (N = 1) Weight: 10000 and 2) Maximum number of N-locus variants (N = 2) Weight: 10.

Discriminatory power and its confidence interval were calculated using Simpson´s index of diversity, as previously described (Hunter and Gaston, 1988) using BioNumerics and the V-DICE diversity calculator from Public Health England available at: http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl.

Results

Description of the outbreak

From November 2013 to March 2014 an increase in the prevalence of S. Typhimurium was noted in the surveillance of the Danish poultry production, where phage type DT41 isolates were found at various stages of the broiler production chain, as well as in a single table egg layer flock and in animal feed (Tables 1 & 2). The table egg layer flock where DT41 was found in November 2013 had a contemporary infection with another *Salmonella* phage type (S. Typhimurium DT40). During the study period, S. Typhimurium DT40 was also found in three other layer flocks (data not shown). A search in DTU Food´s *Salmonella* strain collection going back to 2005 revealed that DT41 had previously been isolated from one of these three farms in 2009, and again in 2010, DT41 was isolated from the farm with the DT40/DT41 infection.

In the broiler production chain DT41 was first found in a broiler farm (A1) from producer A in the start of November 2013 (Table 2). Until the end of March 2014 DT41 and/or RDNC phage types was further isolated

from eight broiler farms (six (A2-A7) and two (B1-B2) from farms linked to producer A and B, respectively). DT41 were also isolated from two broiler breeder farms (D1 and D3, in December 2013 and March 2014, respectively). None of these farms had a previous record of DT41 isolations. In December 2013 an environmental sample taken at the hatchery (D2) was positive for DT41. This hatchery had been receiving eggs from the broiler breeder farm D1. Moreover, farm D1 had delivered one-day-old chickens to broiler farms A2-A7 and B2.

In addition, one DT41 strain isolated from a feed sample taken within the frame of the Danish surveillance of feed production (Anonymous, 2014) was obtained. During the period December 2013 to February 2014 DT41 was isolated, as part of the surveillance programs, from three samples taken at two different abattoirs (F1 and G1) used for slaughtering of broilers from Producer A, although not the specific flocks from farms A2-A7 where DT41 had been isolated during the same period.

Typing of isolates

To further investigate the epidemiology and identify potential sources of the outbreak, 47 DT41 and RDNC isolates were further subtyped using PFGE and MLVA (Figure 1, Table 1). PFGE analysis divided the isolates into four types (types A-D; DI = 0.24 ± 0.15 (95% CI)), with 1-4 bands difference between PFGE types (Figure 1). MLVA-results showed that the five RDNC phage type isolates, were related to the DT41 isolates and they therefore remained in the analysis of the data. On the basis of MLVA results, the DT41/RDNC isolates were split into nine types (DI = 0.65 ± 0.14 (95% CI); Figure 1). Locus STTR9 was found to be identical for all isolates (allele 2) and for STTR3 all but one isolate was identical (0212) - the last isolate had the allele 0112. For STTR5 and STTR10 three (10, 12, 13) and four (8, 9, 10, 12) types were found, respectively. The highest variation was noted for STTR6 where 7 different types were found (10, 11, 12, 13, 14, 15, 16).

The most common MLVA profile was 2-13-12-8-0212 which was isolated from the hatchery, seven broiler farms (six and one from broiler producer A and B, respectively), as well as on two occasions from a slaughterhouse (Figure 1). Merging isolates with only one locus difference resulted in four groups. The most

prevalent group contained isolates from the hatchery (D2), two broiler breeding farms (D1 and D3), broiler farms (A2-A7 and B2), and one of the slaughterhouses (F1) (Figure 1, Table 2). An epidemiological link was established between these units; see the section on the description of the outbreak.

The second group contained isolates from broiler farm A1 and from slaughterhouse G1, differing with at least 2 loci from its closest neighbour (Figure 1). No epidemiological link was found between broiler farm A1 and the other broiler farms. The isolates from the egg layer flock (MLVA profile 2-12-10-10-0212) were found to differ by two loci from its closest neighbor, but had only one loci difference to one isolate from broiler flock B1 from producer B. No epidemiological link could be established between these two occasions, nor to any of the broiler breeding farms. The feed isolate was found to be different from the rest of the isolates (3 loci difference from its closest neighbour) although it shared PFGE type with many of the other isolates.

A search in DTU Food´s *Salmonella* typing database showed that the findings reported in the current study were the first recorded occasions with MLVA type 2-13-12-8-0212, and same was noted for the majority of the single locus variants of this type (data not shown). No human isolates of the most commonly found MLVA types in this study were found in the Danish surveillance system during the same period of time (personal communication, Mia Torpdahl, Statens Serum Institut, Denmark).

Discussion

Salmonella is rarely found in Danish poultry production, and very seldom in broiler breeder flocks (Anonymous, 2014). Nevertheless, reoccurring isolations with S. Typhimurium phage type DT41 has occurred for more than 10 years in particularly the broiler production chain, resulting in the need for expensive and cumbersome actions to be taken by the poultry industry. Previous investigations using MLVA revealed a high diversity in isolates from Danish broiler breeding flocks and it was concluded that no persisting clones of DT41 was present in Danish poultry production, but that the reoccurring infections was due to an outside source (Litrup et al., 2010). However, the instability of the MLVA loci could make it hard

178 to draw correct conclusions from MLVA data (Litrup et al., 2010; Barua et al., 2013; Wuyts et al., 2013;
179 Dimovski et al., 2014). Highest variation has previously been noted for the STTR6 and STTR5 loci, which is
180 consistent with the data generated in the present study where STTR6 was found to be the most variable,
181 followed by STTR10 and STTR5. To handle this expected variation different models have been suggested,
182 e.g. joining isolates that are differing by one loci (Torpdahl et al., 2007) independent of which loci, or more
183 recently, taking the variation of the different loci into account in a model where isolates with identical
184 alleles for STTR3 and STTR9, but with a one allele difference in the more rapidly changing loci STTR5, STTR6
185 and/or STTR10 are merged (Dimovski et al., 2014).
186
187 When joining isolates that differed by one locus into groups, the nine MLVA types for the 47 DT41/RDNC
188 isolates were merged into four groups. This criterion has often been applied to find epidemiologically
189 related strains in outbreak investigations (Torpdahl et al., 2007). The differences within the groups that
190 contained more than one isolate each were due to changes in STTR6 (one group), STTR3 (one group) and a
191 combination of changes in STTR5 and STTR6 (one group). If the variability of the different loci was taken
192 into account, as proposed by (Dimovski et al., 2014) one of the groups were split into two, meaning that
193 one broiler isolate (from farm A1) was no longer linked to other broiler isolates from the same flock and to
194 one slaughterhouse isolate (G1). This seems unlikely as there is a strong epidemiological link between these
195 isolates. This result shows that data need to be interpreted with caution and combining typing data with
196 epidemiological information in order to conclude at the highest level of epidemiological concordance
197 (Struelens, 1996). Focus should especially be given to determination of a natural variation within isolates
198 from the same flock and on isolates found on repeated occasions on the same farm. More discriminatory
199 typing methods such as whole genome sequencing (WGS) would most likely be able to reveal a more
200 accurate relationship between these isolates and thus assist in drawing correct conclusions from the data.
201
202 The convergence between results obtained with MLVA and PFGE was high, although MLVA had a higher DI.
203 There were two MLVA types that contained two different PFGE profiles each, the rest of the MLVA types
204 consisted of isolates with one PFGE type (Figure 1). This high convergence is well recognized in previous

studies, although combining PFGE and MLVA results has been shown to increase the DI (Torpdahl et al., 2007; Broschat et al., 2010; Kurosawa et al., 2012). Again, the application of more discriminatory methods would assist obtaining a correct interpretation of data.

The most commonly found MLVA types in this study have, to the best of our knowledge, seldom been isolated from Danish food and veterinary sources, including poultry. However, as MLVA is a relatively new technique, and not being used on all isolates, the data set used for comparison might not be representative of the true occurrence in the Danish animal population. In addition, when comparing the MLVA data for DT41 isolates from 2013/2014 to previous investigations (Litrup et al., 2010; Barua et al., 2013) it can be noted that there is some overlap in the types found. For example, the same MLVA type 2-12-12-8-0212 isolated from one broiler breeding flock in March 2014 was also found in chicken from a broiler breeding farm in 2009 (Litrup et al., 2010). To the best of our knowledge, these breeder flocks don´t originate from the same farm, but it could be speculated that a persistent infection with this, or similar MLVA types, are established in parts of the Danish broiler breeder production. This persisting clone(s) might then contribute to a continuous spread of DT41 in the production pyramid and a microevolution will lead to a slowly changing genotype, causing variation in e.g. the observed MLVA types. The hypothesis with spread of DT41 from wild birds, as proposed by Litrup et al (2010) and further investigated by Barua et al (2013), has not been addressed in the current study and this, or another outside source such as feed, might still be a possible introduction of the DT41. However, more data on the variation of MLVA types within DT41 from various sources, including wild birds and feed, and a comparison to isolates from previous years are needed to be able to draw more specific conclusions from data.

In conclusion, results from the present study suggest, by using a combination of typing data and epidemiological information, that a spread within the broiler production pyramid had taken place from one broiler breeding flock to seven broiler flocks and further to the abattoir. No typing or epidemiological information could link the other included DT41 isolates from feed, other broiler flocks, or the layer flocks to the outbreak. The source of the sudden increase of *S.* Typhimurium DT41 remains unclear and to

232 investigate this in more detail, further studies using e.g. WGS to obtain a higher discriminatory strength and
233 including isolates from a longer period of time and from various sources are in progress.
234

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240 declare.
241

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310

Figures legends

Figure 1. Neighbor joining tree for the 47 *Salmonella* Typhimurium DT41 and RDNC isolates (stars) divided by MLVA profile, and colored based on PFGE profile. Partitioning is based on a maximum divergence of one locus with MLVA (marked in grey). The sample source with no. of isolates (see Table 1 for an explanation) is shown next to each circle together with MLVA profiles for each group in brackets. The insert shows the PFGE profiles (representative isolates for each profile) compared using the Dice coefficient for similarity and unweighted pair group method with arithmetic averages (UPMGA) for clustering.

319 Table 1. Overview of the 47 included *Salmonella* isolates together with typing data.

Isolate no.	Received date (YYYY-MM-DD)	Type of production	Source[a]	Phage type	PFGE type	MLVA type
2013-60-2066-1	2013-11-13	Broilers	A1	DT41	A	2-12-16-12-0212
2013-60-2066-2	2013-11-13	Broilers	A1	DT41	A	2-12-16-12-0212
2013-60-2066-3	2013-11-13	Broilers	A1	DT41	A	2-12-16-12-0212
2013-60-2066-4	2013-11-13	Broilers	A1	DT41	A	2-12-16-12-0212
2013-60-2089-1	2013-11-19	Broilers	B1	RDNC	C	2-12-10-10-0212
2013-60-2151-1	2013-11-28	Egg layers	C1	DT41	B	2-12-14-10-0212
2013-60-2155-1	2013-11-29	Egg layers	C1	DT41	B	2-12-14-10-0212
2013-60-2155-2	2013-11-29	Egg layers	C1	DT41	B	2-12-14-10-0212
2013-60-2155-3	2013-11-29	Egg layers	C1	DT41	B	2-12-14-10-0212
2013-60-2155-4b	2013-11-29	Egg layers	C1	DT41	A	2-12-14-10-0212
2013-60-2160-1	2013-12-03	Broiler breeders	D1	DT41	A	2-13-11-8-0212
2013-60-2182-1	2013-12-04	Broilers	B2	DT41	A	2-13-12-8-0212
2013-60-2182-2	2013-12-04	Broilers	B2	RDNC	A	2-13-12-8-0212
2013-60-2182-3	2013-12-04	Broilers	B2	DT41	A	2-13-12-8-0212
2013-60-2182-4	2013-12-04	Broilers	B2	DT41	A	2-13-12-8-0212
2013-60-2206-1	2013-12-05	Broiler breeders	D1	DT41	A	2-13-13-8-0212
2013-60-2206-2	2013-12-05	Broiler breeders	D1	DT41	A	2-13-13-8-0212
2013-60-2210-1	2013-12-09	Broilers	A2	DT41	A	2-13-12-8-0212
2013-60-2210-2	2013-12-09	Broilers	A2	DT41	A	2-13-12-8-0212
2013-60-2210-3	2013-12-09	Broilers	A2	DT41	A	2-13-12-8-0212
2013-60-2210-4	2013-12-09	Broilers	A2	DT41	A	2-13-12-8-0212
2013-60-2210-5	2013-12-09	Broilers	A2	DT41	A	2-13-12-8-0212
2013-60-2224-1	2013-12-09	Broilers	A3	DT41	A	2-13-12-8-0212

2013-60-2224-2	2013-12-09	Broilers	A3	DT41	A	2-13-12-8-0212
2013-60-2224-3	2013-12-09	Broilers	A3	DT41	A	2-13-12-8-0212
2013-60-2258-3	2013-12-12	Broilers	B2	DT41	A	2-13-12-8-0212
2013-60-2258-4	2013-12-12	Broilers	B2	DT41	A	2-13-12-8-0212
2013-60-2244-3	2013-12-11	Feed	E1	DT41	A	2-10-15-9-0212
2013-60-2261-1	2013-12-16	Broilers	A1	DT41	A	2-12-16-12-0112
2013-60-2262-1	2013-12-16	Broilers	A4	DT41	A	2-13-12-8-0212
2013-60-2262-2	2013-12-16	Broilers	A4	DT41	A	2-13-12-8-0212
2013-60-2262-3	2013-12-16	Broilers	A4	DT41	D	2-13-12-8-0212
2013-60-2278-1	2013-12-17	Broilers	A5	DT41	A	2-13-12-8-0212
2013-60-2279-1	2013-12-17	Broilers	A2	DT41	A	2-13-13-8-0212
2013-60-2279-2	2013-12-17	Broilers	A2	DT41	A	2-13-12-8-0212
2013-60-2279-3	2013-12-17	Broilers	A2	RDNC	A	2-13-12-8-0212
2013-60-2307-1	2013-12-23	Broilers	A6	DT41	A	2-13-12-8-0212
2014-60-21-1	2014-01-03	Hatchery	D2	DT41	A	2-13-12-8-0212
2014-60-19-1	2014-01-03	Slaughter house	F1	DT41	A	2-13-12-8-0212
2014-60-28-1	2014-01-07	Broilers	A7	DT41	A	2-13-12-8-0212
2014-60-34-1	2014-01-10	Slaughter house	F1	DT41	A	2-13-12-8-0212
2014-60-94-1	2014-01-22	Broilers	B2	RDNC	A	2-13-12-8-0212
2014-60-94-2	2014-01-22	Broilers	B2	RDNC	A	2-13-13-8-0212
2014-60-94-3	2014-01-22	Broilers	B2	DT41	A	2-13-12-8-0212
2014-60-105-1	2014-01-27	Broilers	A1	DT41	A	2-12-16-12-0212
2014-60-224-1	2014-02-12	Slaughter house	G1	DT41	A	2-12-16-12-0212
2014-60-427-1	2014-03-25	Broiler breeders	D3	DT41	A	2-12-12-8-0212

320 [a] Farm for broilers, broiler breeders and egg layers

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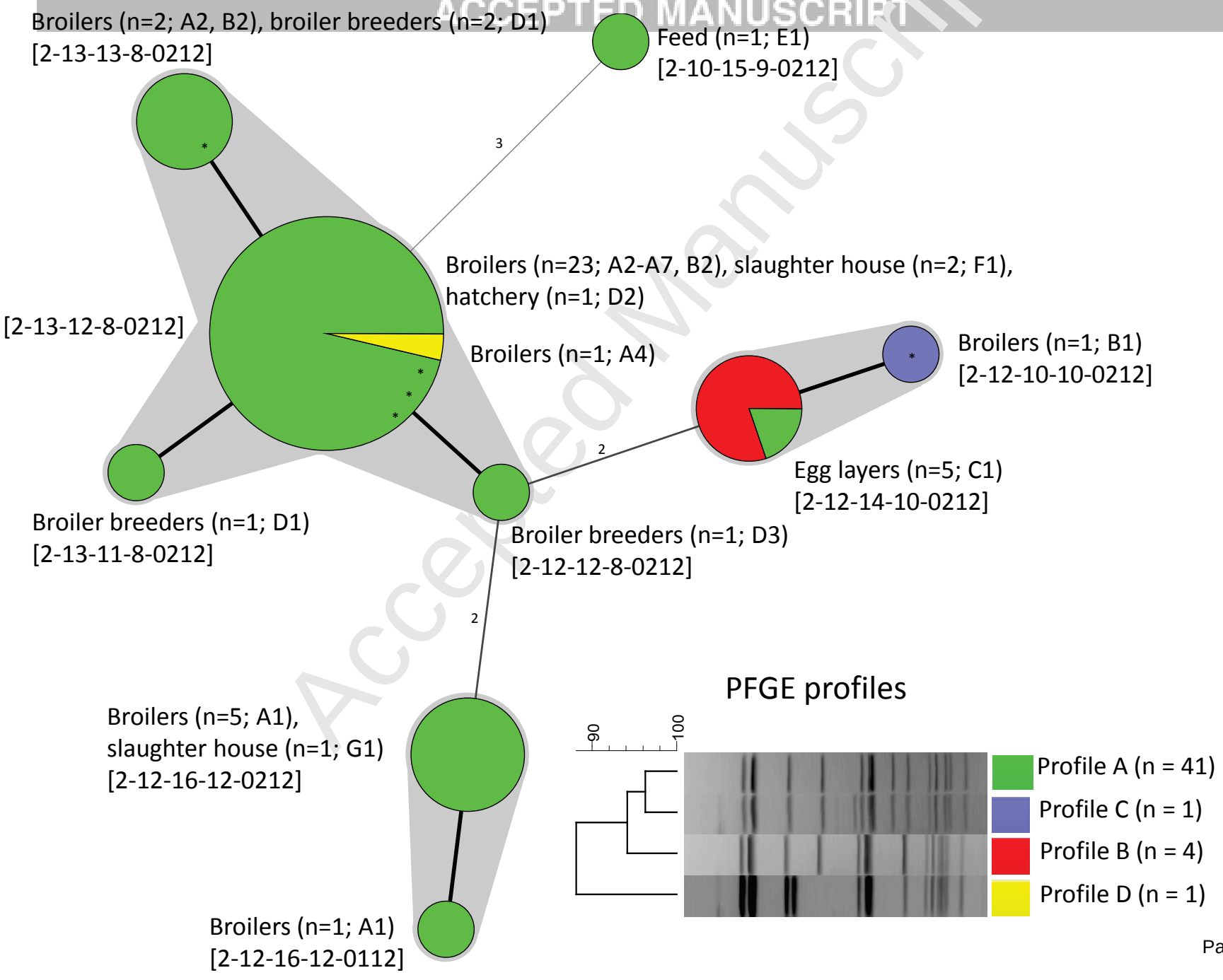

Table 2. Overview of the epidemiological data

Source	Source[a]	No. of events for week no.[b]																					
		46	47	48	49	50	51	52	1	2	3	4	5	6	7	8	9	10	11	12	13	Total	
Broilers	A1	1					1						1										3
	A2					1	1																2
	A3					1																	1
	A4						1																1
	A5						1																1
	A6							1															1
	A7									1													1
	B1		1																				1
	B2				1[c]							1											3
Total broilers		1	1	0	1	2	4	1	0	1	0	1	1	0	0	0	0	0	0	0	0		13
Egg layers	C1			1																			1
Broiler breeders	D1				1																		1
	D3																				1		1
Hatchery	D2								1														1

Total broiler breeders and hatchery		0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1		3
Feed	E1					1																1
Slaughter house	F1								1	1												2
	G1														1							1
Total slaughter house									1	1					1							3
Total no. of events		1	1	1	2	3	4	1	2	2		1	1		1					1		21

[a] Farm for broilers, broiler breeders and egg layers

[b] Bold numbers (in red) represent the outbreak MLVA single loci variant cluster with types 2-13-12-8-0212, 2-13-11-8-0212, 2-13-13-8-0212 and 2-12-12-8-021

[c] Isolates from the same flock were also obtained in week 50, but were regarded as one case as samples were taken in the same house 8 days apart